

Identification of the First Two Virulence Genes of *Moraxella osloensis* in the Slug *Deroceras reticulatum*



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Abstract: The bacterium *Moraxella osloensis* associated with the nematode *Phasmarhabditis hermaphrodita* is a pathogen of slug *Deroceras reticulatum*. Nematode infective juveniles vector *M. osloensis* into the shell cavity of *D. reticulatum*, and the slug is killed within 4-16 days. Gene expression profile of *M. osloensis* in the slug was analyzed and differentially expressed genes were identified using a selective capture of transcribed sequences (SCOTS) technique. Most identified sequences were homologous to other bacteria, and function as putative cell structure, energy metabolism, degradation, translocation, and unknown function proteins. Other identified sequences did not exhibit similarity to any genes or gene products in current databases, and are thus novel. We further evaluated the role of SCOTS identified two genes, *M5* (protein-disulfide isomerase) and *M8* (protein kinase), in the virulence of *M. osloensis* to the slug using mutation and complementation strategy. Mutants were constructed with insertion-deletion strategy by inverse PCR. The full length of *M5* and *M8* fragments were obtained by inverse PCR to complement mutants. Compared to the wild type, the virulence of *M. osloensis* mutants to the slug was markedly reduced and could be complemented. Therefore, we conclude that the *in vivo* expressed genes, *M5* and *M8*, contribute to *M. osloensis* virulence to the slug. This study provided a first glimpse to the gene expression in a mollusk host during bacterial infection, and insights into understanding the mechanism of the bacterial virulence to a mollusk host.

INTRODUCTION

❖ Grey garden slugs, *Deroceras reticulatum*, are regarded as the most serious pests of a wide range of agricultural and horticultural plants (Fig-1).

❖ The nematode *Phasmarhabditis hermaphrodita*, associated with bacterium *Moraxella osloensis* has potential for biological control of *D. reticulatum*.

❖ *P. hermaphrodita* vectors bacterium *M. osloensis* into the shell cavity of *D. reticulatum*, and the bacterium is the main killing agent.

❖ Genes expressed by *M. osloensis* in slugs are likely to play important roles in virulence. Identification of these genes can help in designing improved bacterial strains.

❖ The objective of this study was to identify the virulence genes expressed by *M. osloensis* in slugs after infection.

❖ Selective capture of transcribed sequences (SCOTS) analysis was employed to identify genes expressed by *M. osloensis* in the slug *D. reticulatum* at four days post infection. The roles of SCOTS captured genes were analyzed and evaluated based on the Genomic database and mutagenesis studies.



Fig-1: The left, *D. reticulatum* feeding on oil seed rape seedlings. The right, healthy *D. reticulatum* on left and infected *D. reticulatum* on right with swollen mantle.

HYPOTHESIS

The overall hypothesis is that *M. osloensis* will express genes exclusively in slugs that will help *M. osloensis* to survive, replicate and kill the slug.

DESIGN

Question: Virulence Mechanism / Bacterial Infection
↓
Methods: *In Vivo* Gene Profiles / Gene Identification
↓
Answers: Gene Functions / Mutation - Inactivation

MATERIALS & METHODS

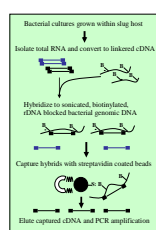
Bacterial Inoculation

❖ Slug *D. reticulatum* was collected from the field and fed on fresh carrots for at least 12 days. Only healthy adult slugs were used in the experiments.

❖ The Pure culture of *M. osloensis* was introduced into the shell cavity of slug *D. reticulatum* via injection.

Selective Capture of Transcribed Sequences

❖ **Preparation of Nucleic Acids:** The *M. osloensis* genomic DNA was prepared. Total RNA was isolated from four days post-infected slugs (*in-vivo*) and from broth cultured mid-log phase bacteria (*in-vitro*). The isolated RNA was converted to cDNA by using random primers with an adapter overhang.

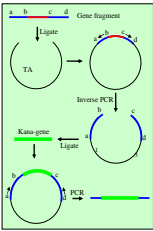


❖ **SCOTS:** Sonicated biotinylated *M. osloensis* genomic DNA was blocked with bacterial rDNA. Prepared cDNA from *in-vivo* or *in-vitro* was added to the DNA-rDNA mixture to hybridize for 24h. Streptavidin coated magnetic particles were used to capture DNA-cDNA hybrids. The captured hybrids were PCR amplified and used for next round of SCOTS.

❖ **Enrichment:** In order to identify the bacterial cDNA molecules that were exclusively expressed in slugs, the cDNA mixtures from *in-vivo* obtained by three rounds of SCOTS were added to biotinylated genomic DNA that had been prehybridized with both rDNA and cDNA obtained by SCOTS from *in-vitro*. Hybridization was carried out for 24h. The hybrids were captured by streptavidin-coated magnetic particles and PCR amplified.

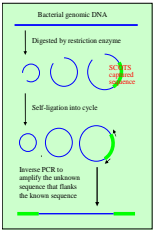
❖ **Analysis of cDNA libraries:** The enrichment subtracted cDNA molecules were cloned into a plasmid vector to generate cDNA libraries. Individual cDNA molecule was screened by hybridization with probes made from cDNA mixtures obtained by SCOTS either from *in-vivo* or *in-vitro*. The screened cDNA molecules were subjected to partial sequence analysis. Sequence analysis was carried out by using BLAST algorithms in NCBI.

Mutation Studies



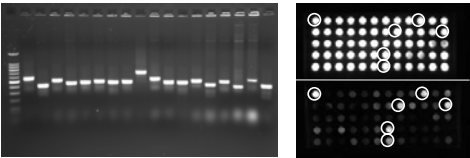
Mutation on the SCOTS identified genes was achieved by insertion-deletion strategy using inverse PCR, in which the kanamycin⁺ gene was used as a selective marker. The resulting product was naturally transformed into wild-type *M. osloensis*. Insertion of the kanamycin⁺ gene through a single recombination event resulted in the disruption of the wild-type gene.

Complementation Studies



The full length of SCOTS identified genes was obtained by inverse PCR strategy. Then the intact gene was cloned into a cloning vector and transferred into the responded *M. osloensis* mutant by electroporation. The *M. osloensis* virulence of wild type, mutants, and complemented mutants was determined by direct infection into the slug *D. reticulatum*.

RESULTS

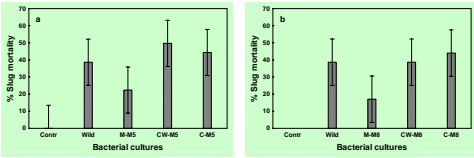


Left: The enrichment subtracted cDNA molecules were cloned into a TA vector, and each individual cloned inserts were PCR amplified to generate subtracted cDNA libraries. Right: Amplified individual inserts were screened by southern hybridization with probes made from SCOTS captured cDNA mixtures either from *in vitro* (probe-*vitro*, below the line) or *in vivo* (probe-*vivo*, above the line) bacterial cultures. The cycled dots were not selected for sequence analysis because they represented the clones that both hybridized to probe-*vitro* and probe-*vivo*. The other dots above the line were chosen for sequence analysis because they represent clones that only hybridized to the probe-*vivo*.

SCOTS identified *in vivo* expressed genes

| Genes | Source of homolog | Possible functions - Pathway |
|-------|--------------------------|--|
| M1 | <i>Streptococcus</i> sp. | Putative surface protein |
| M2 | <i>Mycoplasma</i> sp. | Virable surface lipoprotein |
| M3 | <i>Mycobacterium</i> sp. | Proline rich protein |
| M-HS | | Hypothetical proteins |
| M-HS1 | <i>Psychrobacter</i> sp. | Preprotein translocase subunit SecA |
| M-HS2 | <i>Psychrobacter</i> sp. | Acyl-coA synthetases |
| M-HS3 | <i>Psychrobacter</i> sp. | Acetyl-CoA carboxylase |
| M4 | <i>Lactobacillus</i> sp. | Cell surface protein precursor |
| M5 | <i>Psychrobacter</i> sp. | Protein-disulfide isomerase |
| M6 | <i>Acinetobacter</i> sp. | ATP-binding protein - Energy metabolism |
| M7 | <i>Psychrobacter</i> sp. | Acetyltransferase - Energy metabolism |
| M8 | <i>Psychrobacter</i> sp. | Protein kinase - Ubiquinone synthesis |
| M9 | <i>Psychrobacter</i> sp. | Decarboxylase subunit - Degredation |
| M10 | <i>Acinetobacter</i> sp. | Aldehyde dehydrogenase - Energy metabolism |
| Mos | No hits in NCBI | Unknown |

➤ Analysis was based on database in the GenBank. Genes of M5 and M8 were selected for mutation and complementation studies.



Virulence of different *M. osloensis* cultures to the slug. Compared to the wild type, the virulence of *M. osloensis* mutants was markedly reduced and could be complemented. Contr, Saline; Wild, wild type *M. osloensis*; M-M5, mutants of M5 gene; CW-M5, the plasmid containing the intact M5 gene was transferred into wild type *M. osloensis* as the control of M-M5; C-M5, the complemented mutant of M5 gene; M-M8, mutants of M8 gene; CW-M8, the plasmid containing the intact M8 gene was transferred into wild type *M. osloensis* as the control of M-M8; C-M8, the complemented mutant of M8 gene.

CONCLUSIONS

- ❖ SCOTS identified genes were considered to be over-expressed or exclusively expressed in slugs by *M. osloensis*.
- ❖ Most of the identified sequences are homologous to other bacteria, and function as putative cell structure, energy metabolism, degradation, translocation, and unknown function proteins. Other identified genes do not exhibit similarity to any genes or gene products in current databases, and are thus novel.
- ❖ The sequences reported in this poster have been deposited in the GenBank database (accession no. DQ324260-DQ324276)
- ❖ Two of the identified genes were characterized by mutagenesis studies, and these two genes were confirmed to be virulence genes, which suggests that SCOTS analysis is a suitable tool for the study of gene expression in infected animal tissue.
- ❖ This study provided a first glimpse to the gene expression in a mollusk host during bacterial infection, and insights into the understanding of the bacterial virulence to a mollusk host

FUTURE PROSPECTS

Future research may focus on the understanding of the global gene expression that regulates virulence of bacteria through comparative studies between *M. osloensis* and other related bacteria.

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